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Article

The Role of PIWI and the miRNA Machinery in *Drosophila* Germline Determination

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Summary

Background: The germ plasm has long been demonstrated to be necessary and sufficient for germline determination, with translational regulation playing a key role in the process. Beyond this, little is known about molecular activities underlying germline determination.
Results: We report the function of *Drosophila* PIWI, DICER-1, and dFMRP (*F*ragile X *M*ental *R*etardation *P*rotein) in germline determination. PIWI is a maternal component of the polar granule, a germ-plasm-specific organelle essential for germline specification. Depleting maternal PIWI does not affect OSK or VASA expression or abdominal patterning but leads to failure in pole-plasm maintenance and primordial-germ-cell (PGC) formation, whereas doubling and tripling the maternal *piwi* dose increases OSK and VASA levels correspondingly and doubles and triples the number of PGCs, respectively. Moreover, PIWI forms a complex with dFMRP and DICER-1, but not with DICER-2, in polar-granule-enriched fractions. Depleting DICER-1, but not DICER-2, also leads to a severe pole-plasm defect and a reduced PGC number. These effects are also seen, albeit to a lesser extent, for dFMRP, another component of the miRISC complex.
Conclusions: Because DICER-1 is required for the miRNA pathway and DICER-2 is required for the siRNA pathway yet neither is required for the rasiRNA pathway, our data implicate a crucial role of the PIWI-mediated miRNA pathway in regulating the levels of OSK, VASA, and possibly other genes involved in germline determination in *Drosophila*.

Introduction

In metazoans, primordial germ cells (PGCs) represent the totipotency source that is set aside from somatic lineages early during embryogenesis; this source gives rise to gametes for the reproduction of the species. Elucidation of the mechanisms underlying PGC formation and development is key to understanding the molecular nature of totipotency, the subject of a fundamental question in biology.

With *Drosophila* as a model, considerable progress has been made in identifying genes required for germline specification as well as transcriptional and translational mechanisms required for maintaining the established germline [1]. In many invertebrates and vertebrates, germ cells are specified by a special cytoplasmic region known as the germ plasm. In *Drosophila*, germ plasm, also known as pole plasm, is localized at the posterior of the egg and persists in early embryos during syncytial nuclear divisions. Between the eighth and ninth divisions, 2–3 nuclei migrate into the pole plasm, where they continue to divide, leading to the formation of pole buds [2, 3]. At the tenth nuclear division, the pole buds encapsulate polar nuclei to form PGCs, also known as pole cells [4]. Pole plasm is characterized by the presence of particulate structures, termed polar granules, that are essential for germline determination and posterior patterning [5–9]. Key molecules have been discovered in polar-granule assembly [10–14]; these include molecules involved in the localization and translational regulation of OSK mRNA during oogenesis [15–20]. Translational regulation and the RNAi pathway have also been implicated in polar-granule assembly during oogenesis involving proteins such as VASA and Aubergine that are required for both abdominal patterning and germline determination [18, 21–23]. However, the biochemical activities of pole plasm specific for germline determination remain elusive. Here, we provide evidence that components of the miRNA pathway appear to be present in pole plasm and to play a key role in germline determination.

The miRNA and siRNA pathways represent two distinct yet closely related mechanisms of gene regulation that share several integral components. First, the ribonuclease III enzyme Dicer is involved in generating a small RNA duplex approximately 22 nucleotides in length [24]. Second, one strand of the duplex is loaded into the RISC (*R*NA-*i*nduced *s*ilencing *c*omplex) [25], where it forms a heteroduplex with its target mRNA. A third common and essential component of miRISC and siRISC is a member of the ARGONAUTE (AGO) protein family, whose members contain a PAZ domain capable of binding to small RNAs [26] and a RNase H-like PIWI domain [27]. AGO2 has been shown to be the Slicer of the RNAi pathway in mammals [28–30], and both AGO1 and AGO2 have Slicer activity in flies [31]. PIWI binds to rasiRNAs and has been implicated in both transcriptional and posttranscriptional gene-silencing RNAi pathways [32, 33], although it is not known whether PIWI is also involved in the miRNA pathway. An additional common RISC component may be dFMRP, although its function may be a more regulatory one than the core function of the AGO family proteins [34–37].

The key functional distinction between the miRNA and siRNA pathways lies in the action of their respective RISCs toward target mRNA. In the RNAi pathway,

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siRISC mediates the formation of a perfectly complimentary heteroduplex between an siRNA and its target mRNA. This results in the degradation of the target mRNA by an AGO-family protein in siRISC. In contrast, in the miRNA pathway, miRISC mediates the formation of an imperfect heteroduplex, which typically results in translational repression but can also lead to target mRNA degradation [38–40].

In *Drosophila*, the miRNA and siRNA pathways can be further distinguished by the differential use of two distinct Dicer enzymes: DCR-1 and DCR-2. DCR-1 is necessary for generating miRNAs, whereas DCR-2 is required for generating siRNAs [41]. A recent study has elegantly shown that DCR-1, but not DCR-2, is required for germline stem cell division in the adult *Drosophila* ovary, illustrating their distinct biological functions [42]. Biochemically, human DICER has been shown to interact in vitro with two AGO family proteins through a subregion of the PIWI domain [43]. Furthermore, *Drosophila* PIWI has been implicated in transcriptional and posttranscriptional gene silencing [32] as well as in transposon silencing [44, 45]. However, the potential interaction of PIWI with components of either the siRNA or the miRNA pathway has not been investigated, nor has its biological role beyond the known germline stem cell function [46]. Here, we report that PIWI is a polar-granule component that regulates the expression and localization of the known key polar-granule proteins OSK and VASA in early embryos. Furthermore, we show that PIWI interacts with dFMRP and DCR-1 but not with DCR-2 and that PIWI, dFMRP, and DCR-1, but not DCR-2, are involved in germline fate specification. Together, these results reveal the regulation of germline determinants by PIWI and the miRNA pathway in germline specification.

Results

PIWI Colocalizes with the Polar-Granule Component VASA

Our earlier investigations revealed that *piwi* is maternally required for embryogenesis [46]. To examine the function of *piwi* in embryogenesis, we analyzed PIWI expression in embryos from females expressing a fully functional *myc*-tagged *piwi* transgene [47]. MYC-PIWI is enriched in a crescent at the posterior pole of the embryo during mitotic cycles 1–9 (Figure 1A–1F) and colocalizes with VASA during these cycles in the polar granules (Figures 1G–1I), indicating that it may be a polar-granule component. When pole cells are newly formed at mitotic cycle 10, MYC-PIWI is present in the cytoplasm (Figures 1J–1L). However, MYC-PIWI translocates to the nucleus of all pole cells at the next mitotic cycle (cycle 11; Figures 1M–1O). PIWI is also zygotically expressed in somatic cells at this stage (data not shown). MYC-PIWI remains nuclear in pole cells throughout germ-cell migration and gonadogenesis (Figure S1 in the Supplemental Data available online). This dynamic pattern of PIWI localization indicates its potential role in germline development.

PIWI and VASA Interact in an RNA-Independent Complex in Polar Granules

To verify that PIWI is a polar granule component, we first examined whether PIWI interacts with VASA in a complex. We generated transgenic flies expressing PIWI with a TAP (*t*andem *a*ffinity *p*urification) tag, which has been shown to be an efficient means for isolating protein complexes [48] (Figure S2A). The *TAP-piwi* transgene exhibits proper nuclear localization in adult ovaries (Figure S2B). Furthermore, three independent *TAP-piwi* transgenic lines are able to rescue the $piwi^2$ mutant infertility phenotype (Figure S2C). Therefore, the *TAP-piwi* transgene encodes a TAP-PIWI fusion protein that retains full PIWI function.

We enriched for polar-granule components of embryos from *TAP-piwi* females by sucrose-gradient fractionation of 0–2 hr embryonic lysates by using VASA as a marker for fractions containing polar granules or their substructures (data not shown). To determine if PIWI is in a complex with VASA, we immunoprecipitated TAP-PIWI complexes from VASA-enriched fractions. Western-blot analysis of the immunoprecipitated complex indicates that VASA is associated with PIWI (Figure 1P). This result was confirmed by reciprocal immunoprecipitation of sucrose-gradient-fractionated 0–2 hr embryonic lysates with anti-VASA antibody, which coimmunoprecipitated PIWI (Figure 1Q). These data are consistent with the finding in mice that two PIWI homologs, MIWI and MILI, also interact with the Mouse Vasa Homolog [49]. The PIWI-VASA interaction further demonstrates that PIWI and VASA are in a protein complex. Because VASA is predominantly localized in polar granules, these data, together with the immunofluorescence colocalization data, implicate PIWI as a polar-granule component interacting with VASA.

To investigate whether the PIWI-VASA interaction is RNA dependent, we performed coimmunoprecipitations with RNase A to degrade the RNA and with RNaseOUT to preserve the RNA. The RNase A treatment did not detectably affect the efficiency of coimmunoprecipitation (Figure 1P with RNase A treatment). These results suggest that the PIWI-VASA interaction does not depend on RNA as a mediator.

To further verify that PIWI is a polar-granule component, we then examined whether PIWI can follow polar granules to ectopic sites. Previously, it has been shown that when the 3′ UTR of *oskar* mRNA is replaced by the *bcd* mRNA 3′ UTR that contains the anterior localization sequence, the resulting *osk-bcd* mRNA becomes localized to the anterior pole of the embryo, and this localization produces anteriorly localized OSK that induces the formation of fully functional polar granules at the anterior pole [50]. We found that embryos produced by females containing the *osk-bcd* and *myc-piwi* transgene indeed contain MYC-PIWI that is recruited by OSK to the anterior pole where they colocalize, in addition to their normal localization at the posterior pole (Figure S3). These results further indicate that PIWI is indeed a polar-granule component.

Maternal PIWI Dose Determines PGC Number

To test the potential role of PIWI in embryonic germline development, embryos depleted of maternal PIWI were collected from females containing $piwi^1$ germline stem cell clones crossed to wild-type males. Germ cells in the embryos were visualized by staining with VASA antibodies (Figure 2). In the absence of maternal PIWI, we observed a dramatic reduction in the number of pole

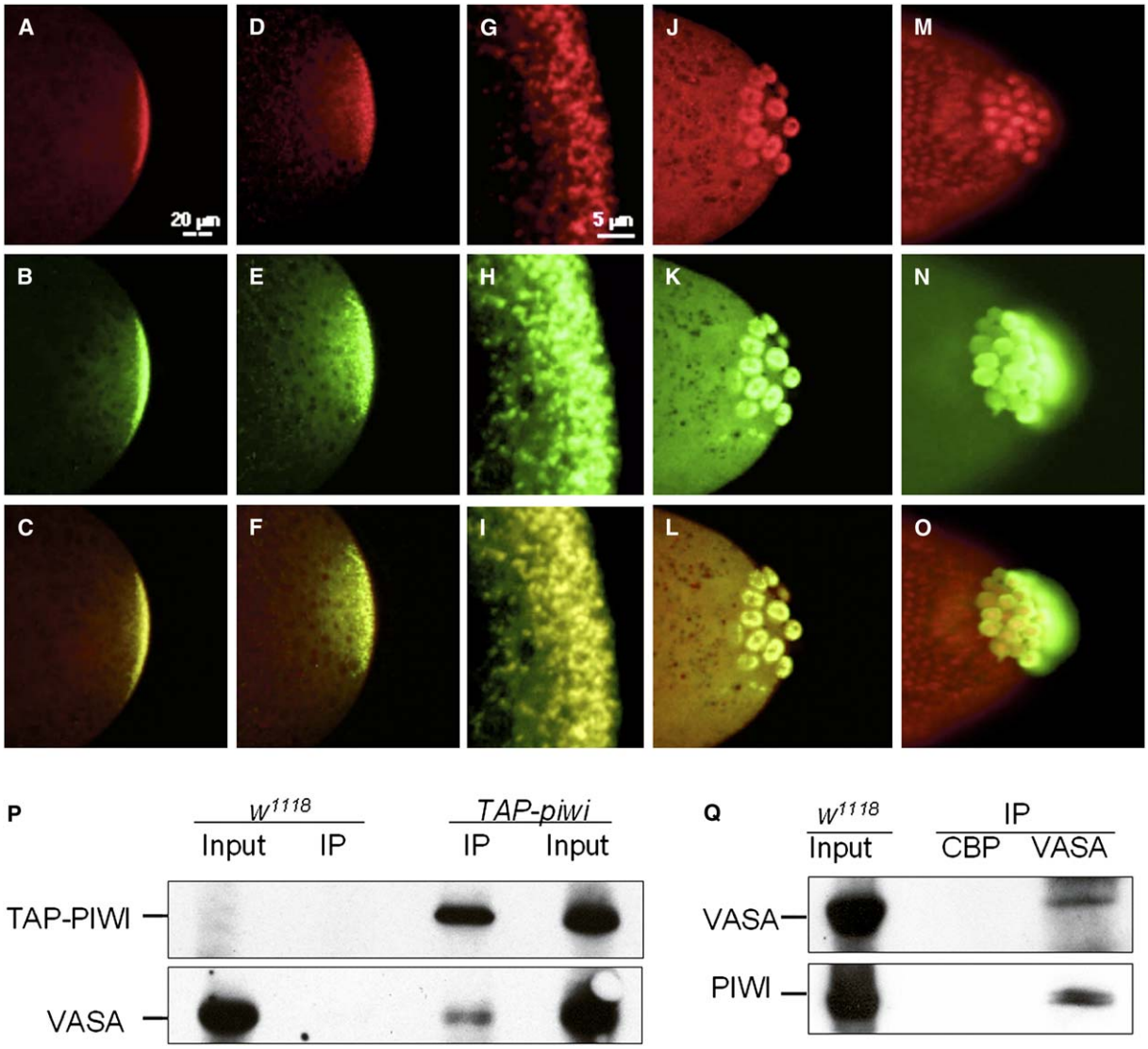

Figure 1. PIWI Is a VASA-Interacting Polar-Granule Component with a Dynamic Behavior during Embryonic Germline Development

Confocal micrographs of wild-type embryos stained with MYC-PIWI (red) and VASA (green) antibodies. (A–F) Images of cycle-2 (A–C) and -5 (D–F) embryos reveal posterior colocalization of PIWI and VASA in a crescent. (G–I) Images of a cycle-8 embryo reveals colocalization of PIWI and VASA to polar granules. (J–L) Image of a cycle-10 embryo reveals PIWI colocalization with VASA in the cytoplasm of the newly formed pole cells. (M–O) PIWI is translocated to the nucleus of pole cells at cycle 11. In all panels, posterior is to the right. The bar in (A) is for panels (A)–(F) and (J)–(O), and that in (G) is for (G)–(I). (P) Two independent western blots containing equal aliquots of content from the same coimmunoprecipitation (co-IP) experiment showing co-IP after sucrose-gradient enrichment of TAP-PIWI and VASA. TAP-PIWI complexes are co-IPed from the fractionated and RNase-A-treated 0–2 hr *Tap-piwi* embryonic lysate (*Tap-piwi* Input) via calmodulin-binding peptide (CBP) antibody. The co-IP was also conducted with the similarly fractionated 0–2 hr embryonic lysate from w^{1118} females as a negative control. The top blot is stained for CBP to detect TAP-PIWI. The bottom blot is stained with anti-VASA antibody. (Q) Two independent western blots containing aliquots of content from the same co-IP experiment showing co-IP after sucrose-gradient fractionation. Input is 0–2 hr w^{1118} embryonic lysate, and VASA complexes are co-IPed with VASA antibody, with CBP antibody as a negative control. The top blot is stained for VASA, and the bottom blot is stained for PIWI.

cells formed. The PIWI-depleted embryos contained on average 7.5 ± 5 pole cells (n = 500) at mitotic cycle 14, in contrast to the 24 ± 5 pole cells (n = 100) in wild-type embryos at the same stage (Figures 2A and 2B). Additionally, 33% of the maternally *piwi*-depleted embryos were devoid of pole cells (Figure 2A). Moreover, a paternal copy of $piwi^{+}$ does not rescue the defect in pole-cell formation. These results indicate that PIWI is required as a maternal component for pole-cell formation.

The small number of pole cells formed in some embryos deficient in PIWI could be due to the residual activity of *piwi* because $piwi^{1}$ is a strong but not necessarily null mutant [46]. Alternatively, it may reflect a redundancy of the maternal function of the *piwi* family of genes.

The maternal effect of *piwi* on pole-cell formation is similar to that of posterior-group genes required for pole-plasm assembly and pole-cell formation [11]. However, PIWI-depleted embryos display no detectable abdominal defects, as judged by their normal embryonic segmentation pattern, Engrailed protein expression, and larval cuticle pattern (Supplemental Results and

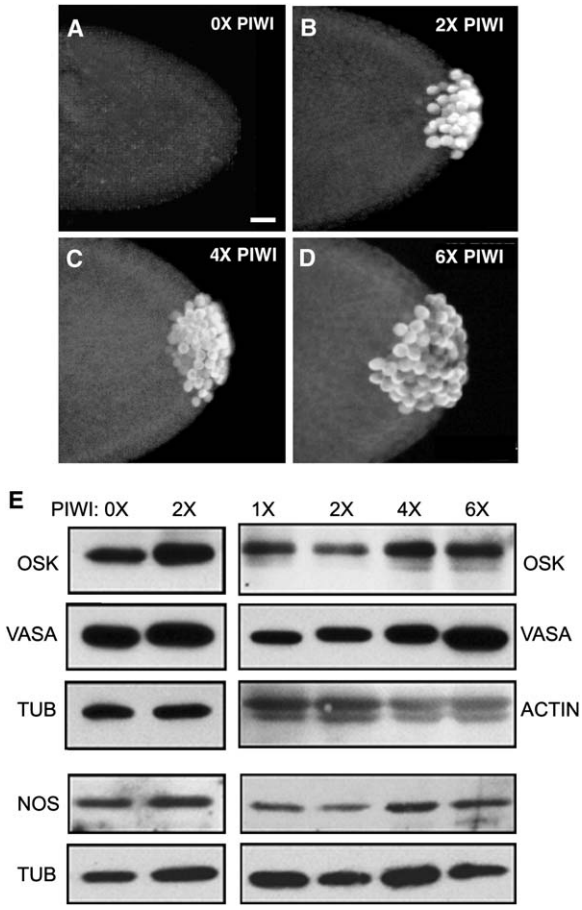

Figure 2. Maternal PIWI Is Required for Pole-Cell Formation in a Dose-Dependent Manner, with Its Overexpression Increasing the Levels of OSK, VASA, and NOS in Early Embryos

Confocal images of embryos from wild-type females carrying zero (A), two (B), four (C), and six (D) copies of *piwi* and stained with VASA antibodies to visualize pole cells. The number of pole cells in (C) and (D) is approximately 2X and 3X of that in (B). The bar in (A) denotes 25 μm for (A)–(D). For quantification, see Figure S5A. (E) Western blots of lysates from 0–2 hr embryos containing increasing maternal loads of PIWI. Genotypes of females that produce embryos are as follows: OX = $piwi^1/piwi^1$ maternal germline, 1X = $piwi^1$/Cyo, 2X = w^{1118}, 4X = two copies of TAP PIWI in a w^{1118} background, 6X = four copies of MYC-PIWI in a w^{1118} background. Blots were probed for OSKAR, VASA, and NANOS; probing for β1-tubulin (TUB) and actin and staining for Ponceau (not shown) were used as loading controls.

Figure S4). Therefore, *piwi* does not appear to be required for abdominal patterning of the embryo. However, it is also possible that *piwi* mutants produce a very weak posterior group phenotype that was not detected by our assays; pole cells are more sensitive than the abdomen to defects in pole-plasm assembly [51].

Given the critical role of *piwi* in pole-cell formation, we further tested whether PIWI represents a dosage-dependent mechanism. To this end, we investigated whether increasing the maternal dose of PIWI increases the number of pole cells. Indeed, cycle 14 embryos from females containing four and six copies of *piwi* contain 50 ± 7 and 66 ± 7.5 pole cells (n = 50 each), respectively, approximately doubling and tripling the number of pole cells observed in the wild-type embryos (24 ± 5). Moreover, the increase in the number of pole cells occurs without a corresponding increase in the number of somatic cells (Figures 2C and 2D; Figure S5A). This increase could be due to either an increase in the initial number of pole cells or their increased mitotic rates, or both. Notably, the 4X maternal *piwi* phenotype is strikingly similar to the effect on the number of pole cells previously observed in 4X maternal *osk* embryos [50] (Figure S5B). However, in contrast to *osk*, increasing maternal *piwi* gene dosage does not cause defects in either abdominal patterning or embryonic polarity, further supporting the assertion that *piwi* is largely dispensable in abdominal patterning. These results indicate that, at least within the tested range, the dose of maternal PIWI has a specific and linear effect on determining the number of pole cells.

PIWI Is Insufficient to Induce Ectopic PGC Formation

The linear dependence of pole-cell number on the maternal PIWI dose demonstrates the important role of PIWI in germline determination. To test whether PIWI alone is sufficient to allow determination of the germline, we expressed maternal PIWI in the anterior region of the embryo. This was achieved with a functional *myc-piwi* transgene in which the 3′ UTR of *piwi* was replaced by the 3′ UTR of *bcd* for anterior localization of the mRNA (Supplemental Results and Figure S6). In nearly 100% of early embryos (n = 700), MYC-PIWI was anteriorly localized (Figures S7A, S7C, S7E, and S7G). However, none of the four examined polar-granule components, OSK, NANOS (NOS), VASA, and GERM CELL-LESS (GCL), is colocalized with MYC-PIWI at the anterior (Figures S7B, S7D, S7F, and S7H), indicating that MYC-PIWI alone is insufficient to recruit required polar-granule components to the anterior of the embryo and that, consequently, PIWI is insufficient to induce formation of pole cells at the ectopic anterior site. Moreover, the anterior of these embryos at cycle 10–11 is morphologically normal, with no discernable pole bud or pole cell (Figures S7I–S7K). This further indicates that PIWI alone is insufficient to induce pole-cell formation independent of polar granules. Finally, no anterior patterning defect or embryonic lethality is associated with the anterior MYC-PIWI localization, again confirming that PIWI does not play a role in embryonic patterning. These results are in contrast with embryos produced by females containing the *osk-bcd* transgene [50], indicating that PIWI is necessary but not sufficient for the formation of the pole plasm and pole cells. Moreover, together with the recruitment of PIWI by OSK to the anterior pole (Figure S3), these results suggest that PIWI is downstream of OSK and VASA in the polar-granule assembly process. Despite this, it is entirely possible that the assembly pathway is different from the regulatory pathway and that PIWI may regulate the expression or localization of OSK and VASA in the pole plasm.

Maternal Dose of PIWI Regulates the Levels of OSK and VASA in Early Embryos

Previous genetic experiments led to the proposal that OSK is the rate-limiting germline determinant that regulates pole-cell numbers in a dose-dependent manner [50], yet our data indicate that PIWI also behaves as a rate-limiting factor. To resolve this apparently

paradoxical relationship between PIWI and OSK in regulating pole-cell numbers, we examined the level of OSK in embryos laid by females that carry 0, 1, 2, 4, and 6 copies of *piwi* genes. To further examine how various doses of maternal PIWI affect pole-plasm components downstream of OSK, we also examined the level of VASA, the most downstream component of the pole-plasm assembly before bifurcation into the abdominal and germline pathways [10, 12], and NANOS (NOS), a factor critical for abdomen formation [52]. Interestingly, although OSK, VASA, and NOS levels are not affected in embryos with 0–2X maternal PIWI, they increase as the maternal PIWI dose increases from 2X to 4X and 6X (Figure 2). The quantification of the levels of these proteins (Table S1) suggests a general trend of overexpression of OSK, VASA, and NOS in the presence of greater-than-wild-type levels of PIWI. Two conclusions can be derived from these results. First, even though PIWI is downstream of OSK and VASA in the assembly process, it can regulate the level of OSK and VASA and, to some extent, NOS, in early embryos. This regulation is not required for expression of OSK, VASA, or NOS but can enhance their expression when PIWI is overexpressed. This could be due to the redundancy between PIWI and its homologs, such as Aubergine, in regulating OSK, and possibly VASA and NOS expression, so that lack of or insufficient PIWI can be compensated by its homologs, yet overexpression of PIWI will sufficiently increase the total regulatory activity of these proteins toward stimulating OSK and VASA translation. Second, the fact that PIWI depletion severely affects pole-cell formation without affecting OSK, VASA, or NOS expression suggests that PIWI has an additional function required for pole-plasm localization and maintenance as well as germline determination.

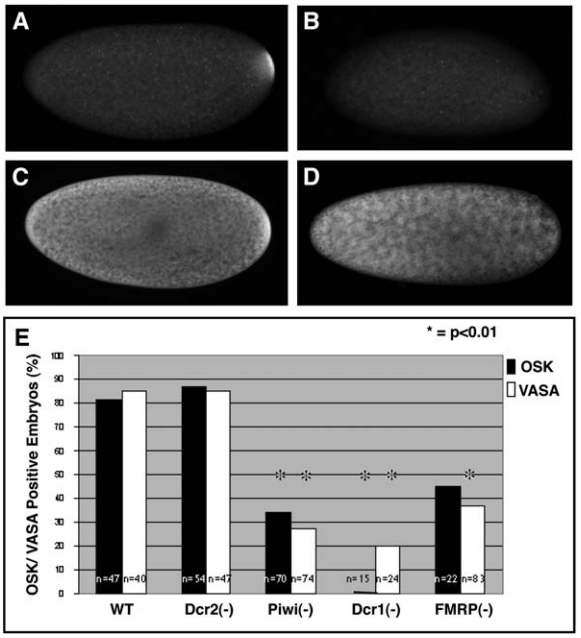

Figure 3. The Effect of Depleting Maternal PIWI, DCR-1, DCR-2, and dFMRP on Pole Plasm

(A and B) Two PIWI-depleted embryos with (A) and without (B) pole plasm as indicated by OSK staining. (C and D) Two PIWI-depleted embryos with (C) and without (D) pole plasm as indicated by VASA staining. (E) The percentage of embryos that are depleted of maternal PIWI, DCR-1, DCR-2, or dFMRP but have pole plasm as indicated by proper pole-plasmic VASA or OSK staining. Starred data are statistically significantly different from the wild-type data, with a p value of less than 0.01.

The Localization of OSK and VASA Is Severely Affected in Embryos Depleted of Maternal PIWI

To confirm that PIWI depletion does not affect OSK and VASA expression, we examined their expression and localization in egg chambers containing *piwi*^{1} mutant germline clones. These clones are identified by the absence of GFP staining in nurse-cell nuclei (Figure S8). We examined the expression and posterior localization of OSK, TUD, and VASA from stage 8–12 of oogenesis because these processes mainly occur between stages 8 and 10b and are completed by stage 12 [53–55]. Both the expression and posterior localization of OSK, TUD, and VASA are apparently normal in all stage-8 to -12 egg chambers with a *piwi*-deficient germline (for OSK, n = 19; TUD, n = 14; VASA, n = 18; Figure S8). These results suggest that PIWI has no obvious effect on either the expression or localization of OSK, TUD, and VASA during oogenesis.

To test whether PIWI is required in embryos for pole-plasm formation in addition to its redundant regulation toward OSK, VASA, and NOS expression, we examined whether pole-plasmic localization of OSK, TUD, and VASA is affected in embryos depleted of maternal PIWI. Of these embryos, 73% show no detectable VASA in the posterior pole (n = 74; Figure 3). The discrepancy between this and the percentage of PIWI-depleted embryos with no pole cells is probably due to the difficulty in detecting significantly reduced VASA signal at the posterior or to the fact that this is an independent sample of depleted embryos. The rest show residual VASA localization in the posterior pole. Similarly, 66% of maternally PIWI-depleted embryos show no detectable OSK in the posterior pole (n = 70; Figure 3). The rest show residual OSK localization in the posterior pole. Given that OSK and VASA are present at normal levels in these embryos (Figure 2), these results suggest that maternal PIWI is required for maintaining the localization of OSK and VASA and, likely, polar granules in early embryos, in addition to its dispensable role in regulating OSK, VASA, and NOS expression. The residual OSK and VASA staining observed in some embryos either reflects the hypomorphic nature of the *piwi*^{1} allele or partial redundancy of PIWI with respect to its requirement for OSK and VAS expression.

The biological significance of OSK and VASA upregulation by increased PIWI dose has been indicated by the fact that the dose of maternal PIWI determines the number of pole cells (Figure 2). To further establish this correlation, we examined whether the presence of residual polar granules, marked by VASA localization, in the maternally PIWI-depleted *piwi* embryos leads to the ability of those embryos to form a small number of pole cells. We reasoned that, were this the case, then only those *piwi* mutant embryos with posterior VASA localization should give rise to pole cells, whereas *piwi* mutant embryos lacking VASA localization would not form pole cells. We generated maternally depleted *piwi*^{1} embryos bearing a functional VASA-eGFP fusion protein [56],

allowing us to collect real-time data on VASA localization in individual embryos from the earliest syncytial stage to the cellular blastoderm stage via in vivo time-lapse fluorescence imaging. As predicted, all embryos displaying positive VASA posterior localization form a small number of pole cells (n = 9), whereas none of the embryos lacking posterior VASA localization ever form pole cells (n = 37; Movies S1 and S2). These results further suggest that the requirement of PIWI for maintaining the localization of VASA and other polar-granule components in early embryos is important for pole-cell formation.

PIWI Interacts with Components of the miRNA Pathway in an RNA-Independent Manner

We then further explored the biochemical mechanism underlying the function of PIWI in regulating OSK and VASA expression and maintaining their localization in early embryos. Given that PIWI family members are known to interact directly with DICER and act as essential components of RISC [27, 43, 57, 58], we examined whether PIWI also interacts with known components of RISC, DCR-1, DCR-2, or dFMRP [35, 36] in the pole plasm. If so, PIWI may act through a small RNA-mediated mechanism. To identify potential PIWI interactors in pole plasm, we enriched for polar-granule components by fractionating 0–2 hr total embryonic lysates on a sucrose gradient by using VASA as a marker for polar granules or their substructures containing VASA, as reported in Figure 1. Western-blot analysis of individual fractions indicates that TAP-PIWI, DCR-1, DCR-2, and dFMRP cosediment in the VASA-enriched fractions (data not shown). These results indicate that DCR-1, DCR-2, and dFMRP may also be enriched or specifically present in polar granules.

To further investigate whether PIWI interacts with DCR-1, DCR-2, and dFMRP, we immunoprecipitated TAP-PIWI from the TAP-PIWI fractions in which these factors cosediment. Western-blot analysis of the immunoprecipitated complexes from the same experiment as that presented in Figure 1P reveals that dFMRP and DCR-1, but not DCR-2, coimmunoprecipitate with TAP-PIWI (Figure 4A). These results indicate that PIWI interacts with the Dicer that is specifically required for the miRISC (DCR-1) as well as with a core component of the RISC (dFMRP) but does not interact with the siRNA-specific Dicer (DCR-2). Additionally, no differences in coimmunoprecipitation efficiency were noted in the presence of RNase A, indicating that the interactions among PIWI, DCR-1, and dFMRP are not dependent on RNA.

To test whether the interaction occurs in polar granules, we used DCR-1 and dFMRP antibodies to probe the western blot that is presented in Figure 1Q and that contains PIWI in an anti-VASA coimmunprecipitated complex. Indeed, DCR-1 and dFMRP are also detected in a complex with VASA, similar to PIWI (Figure 4B). Therefore, PIWI may achieve its function in regulating OSK, VASA, and NOS expression and in germline fate determination via an miRNA-mediated mechanism in the germ plasm.

Maternal DCR-1 and dFMRP Are Involved in PGC Formation

To test the hypothesis that PIWI may achieve its function in germline fate determination via an miRNA-mediated

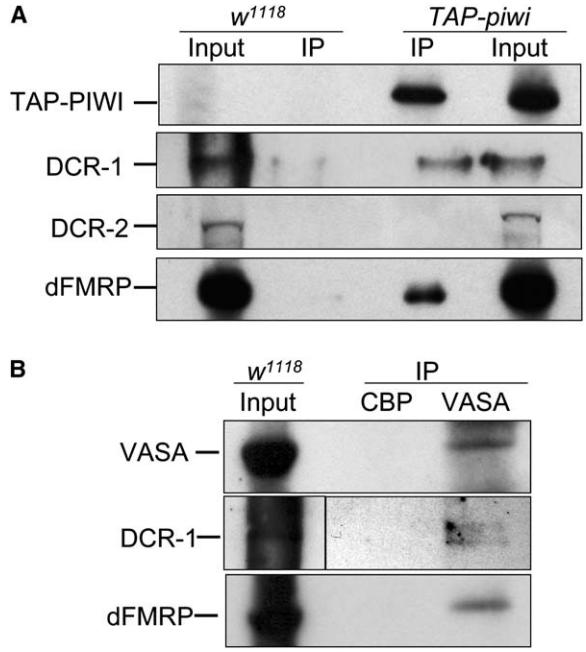

Figure 4. PIWI Interacts with Integral Components of the miRNA Machinery

(A) Western blots showing co-IP of TAP-PIWI with dFMRP and DCR-1 but not DCR-2 in fractions enriched for polar granules and their components by sucrose-gradient sedimentation as described in Figure 1P. Input was 0–2 hr w^{1118} or *Tap-piwi* embryonic lysate, and TAP-PIWI complexes were IPed with the CBP antibody. The western blots probed for TAP-PIWI, DCR-2, and dFMRP represent three independent blots containing equal aliquots of content from the same co-IP experiment as that in Figure 1P. The DCR-1 blot is from a separate co-IP experiment that exhibited the same level of TAP-PIWI complex precipitation.

(B) Three independent western blots containing aliquots of content from the same co-IP experiment as that described in Figure 1Q. Shown is co-IP of VASA with dFMRP and DCR-1 after sucrose-gradient enrichment. Input was 0–2 hr w^{1118} embryonic lysate, and VASA complexes were IPed with the VASA antibody. The blots are stained for VASA, DCR-1, and dFMRP, respectively. For the DCR-1-stained western blot, the input lane is shown with a shorter exposure time than the rest of the blot.

mechanism in the germ plasm, we examined whether DCR-1 and dFMRP are involved in germline determination. We collected early embryos depleted of maternal DCR-1 or dFMRP from females containing *dcr-1*$^{-}$ or *dfmr*$^{-}$ germline stem cell clones crossed to wild-type males and observed a drastic reduction in the number of pole cells formed in mutant embryos, similar to PIWI-depleted embryos (Figures 5A–5D). Specifically, 68% of dFMRP-depleted embryos are devoid of pole cells (n = 443), whereas the remaining 32% of embryos contain 22 ± 2 pole cells. Likewise, 70% of DCR-1-depleted embryos are devoid of pole cells (n = 100), whereas the remaining 30% of embryos contain 16 ± 3 pole cells. In contrast, *dcr-2* mutant embryos from homozygous *dcr-2* females mated with homozygous *dcr-2* males develop a normal number of pole cells (25 ± 6, Figures 5E and 5F). Collectively, these results indicate that *dcr-1* and *dfmrp*, but not *dcr-2*, are required for germ-cell formation.

This requirement could reflect either an indirect consequence of the function of DCR-1 and dFMRP in

embryonic mitosis or their direct role in germline specification, or both. The indirect role should be seriously considered particularly given that the number of pole cells in the dFMRP-deficient embryos displays a nearly "all-or-none" bipolar distribution. The formation of pole cells in 30%–32% of embryos deficient in DCR-1 or dFMRP is unlikely to be due to the residual activity of these genes because the *dcr-1* and *dfmr* mutants used are likely null [41, 59]. To distinguish between the role of DCR-1 and dFMRP in embryonic mitosis versus germline specification, we first examined the mitosis of DCR-1- and dFMRP-deficient embryos at each specific early embryonic cell cycle. Consistent with the mitotic function, 37% of DCR-1-depleted embryos (n = 100) were arrested at embryonic cycle 1 or were unfertilized, and 9% were arrested prior to embryonic cycle 8. Likewise, 59% of dFMRP-depleted embryos (n = 200) were either arrested at cycle 1 or were unfertilized, and 9% were arrested before cycle 8 (Figure S9A). In addition, severe defects in chromosomal segregation, nuclear distribution, and supernumerary mitoses were evident in DCR-1- and dFMRP-deficient embryos (Figures S9A–S9D). Despite this, among mitotically normal DCR-1-deficient embryos with nuclei present in the posterior pole region at cycle 9–14, 50% of them had no pole cells, and 22% contained fewer than ten pole cells (Figures 5A and 5B). Only 27% contained approximately normal numbers of pole cells. These data suggest that, in addition to its mitotic function, DCR-1 has a direct role in germline specification. Remarkably, such combined mitotic and germline defects were also observed in PIWI-depleted embryos at very similar frequencies (data not shown), further supporting the idea that DCR-1 and PIWI are involved in the same embryonic processes.

Similar to DCR-1 and PIWI, dFMRP also appears to have a role in germline specification, albeit to a lesser extent. Among mitotically normal dFMRP-deficient embryos at cycle 9–14, only 3% of them had no pole cells (Figure 5C and 5D), and 20% of them contained fewer than ten pole cells, yet 77% displayed approximately normal numbers of pole cells (n = 30). Independent of these considerations, the maternal-depletion phenotypes of *dcr-1*, *piwi* and, to a lesser extent, *dfmrp*, in conjunction with the coimmunoprecipitation data of PIWI, DCR-1, dFMRP, and VASA, suggest that the miRNA pathway, but not the siRNA or rasiRNA pathway, is involved in *Drosophila* germline fate determination.

To further explore the roles of DCR-1 and dFMRP in germline determination, we examined whether polar granules are present in embryos depleted of DCR-1, DCR-2, or dFMRP by using OSK and VASA as polar-granule markers (Figure 3). Consistent with our quantification of pole-cell numbers, VASA localization is unaffected in the DCR-2-depleted embryos but is abolished in 80% of DCR-1-depleted embryos (n = 24). Among dFMRP-depleted embryos, 63% of them have no detectable VASA localization (n = 83). This observation is similar to the finding that 73% of PIWI-depleted embryos had undetectable VASA localization at the posterior pole (n = 74). Moreover, all of these findings are significantly different from those observed in the wild-type embryos. Very similar effects were also observed for OSK localization (Figure 3). Because the pole plasm is formed prior to stage 12 of oogenesis and is independent of

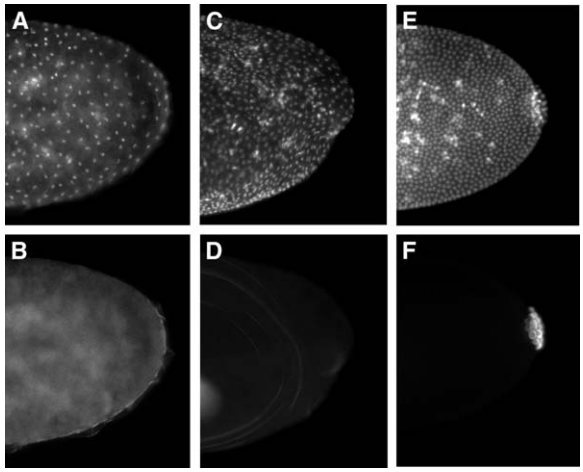

Figure 5. Maternal dFMRP and DCR-1, but not DCR-2, Are Involved in Pole-Cell Formation

DAPI (A, C, and E) and VASA antibody (B, D, and F) staining showing the posterior half of the mitotically normal embryos depleted of maternal DCR-1 (cycle 10 [A and B]), dFMRP (cycle12 [C and D]) or DCR-2 (cycle 14 [E and F]). DCR-1- and dFMRP-depleted embryos contain no pole cells, yet DCR-2-depleted embryos still form a normal number of pole cells.

embryonic mitosis, these data indicate that maternal DCR-1, PIWI, and dFMRP, but not DCR-2, have a direct role in germline determination and that this role is correspondingly independent of embryonic mitosis. Given that pole-cell formation is normal in many dFMRP-depleted embryos, the role of dFMRP in germline determination would appear to be largely redundant.

Discussion

It has been nearly a century since the discovery of germ plasm and its function in germline fate determination in diverse organisms. In recent decades, the components and assembly of the polar granule in *Drosophila* and its equivalent in *C. elegans* have been effectively explored. Translational regulation has also been implicated in pole plasm for abdominal patterning and germline determination. In addition, *germ cell-less* (*gcl*) and mitochondrial large-subunit ribosomal RNAs (mtlr RNAs) have been shown to be required for germline determination [60–62]. However, the biochemical activities of these molecules remain largely unknown. Our study identified PIWI and likely the miRNA machinery as a germ-plasm regulatory activity that is involved in germline fate determination.

Germ-plasm assembly occurs in a stepwise fashion [12]. Step 1 involves the transport of polar granule materials to the posterior end of the oocyte during oogenesis, a process that involves a microtubule-based transport system as well as genes such as *cappuccino* [53, 55] and *staufen* [54, 63]. Step 2 is the assembly of polar-granule components at the posterior end, a process that is almost concurrent with the transport and that is completed by stage 12 of oogenesis. A critical component for the assembly is OSK, which determines the pole-cell number in a dose-dependent manner and has the ability to recruit VASA and TUD as well as to induce pole-cell formation at ectopic sites within the embryo

[50]. Three lines of data suggest that PIWI is downstream of OSK, TUD, and VASA in the assembly process. First, OSK, TUD, and VASA appear to assemble normally into the pole plasm in PIWI-depleted developing oocytes. Second, PIWI cannot recruit OSK or VASA ectopically to the anterior pole, yet OSK can recruit PIWI to the anterior pole. Third, OSK, TUD, and VASA all have both germline determination and posterior-patterning functions, but PIWI does not appear to have a detectable function in patterning.

Although the assembly of polar-granule components occurs in a hierarchical fashion, there is growing evidence for interactions between polar-granule components beyond what is required for assembly. For example, a regulatory relationship between *nanos* and *tudor* has been reported. In *nanos* mutant embryos, both TUDOR levels and the number of pole cells increase [64]. Other experiments suggest that the presence of *mtlr*RNA in the polar granules is required for stabilization of the polar-granule components VASA, GCL, *nos* mRNA, and *pgc* mRNA [65]. The regulatory function reported here for PIWI toward OSK, VASA, and NOS further supports the interplay and interdependency among pole-plasm components. A previous study implicates *osk* as a rate-limiting factor for all aspects of pole-plasm function [50]. Our results suggest that PIWI, likely working through the miRNA pathway, is also a limiting factor for germ-cell formation. This function of PIWI is likely achieved via regulation of the levels of OSK, TUD, and VASA, and possibly that of other polar-granule components, in a dose-dependent fashion (Figure 2).

The regulation of PIWI toward the expression of OSK, TUD, VASA, and NOS appears to be dispensable; PIWI-deficient oocytes and early embryos do not display detectable defects in their expression of OSK, TUD, VASA, and NOS. This redundancy is likely due to an overlapping function of PIWI with other proteins involved in the RNAi pathway and/or colocalized in nuage during oogenesis; such proteins might include Maelstrom [15, 16], Armitage [20], and Aubergine [17, 18, 23]. Among these proteins, Aubergine, a close homolog of PIWI, is a known polar-granule component in early embryos [17, 18]. It regulates the translation of OSK during oogenesis and is required for both pole-cell formation and posterior patterning during embryogenesis [18, 20].

It is intriguing that PIWI regulates OSK and VASA expression yet does not display a posterior-patterning phenotype. This function is different from that of Aubergine, so it is possible that PIWI and Aubergine each have their own regulatory targets in addition to OSK and VASA. The PIWI targets may be specifically involved in maintaining polar-granule localization and may not be subject to Aubergine regulation, whereas Aubergine targets might be involved in both germline determination and posterior patterning. In support of this possibility, it has recently been shown that the generation of certain rasiRNAs shows varying dependencies on PIWI and Aubergine [33]. The regulation of PIWI toward its specific target genes may be activated during oocyte maturation, similar to the oocyte maturation-dependent activation of RNAi as observed for *aubergine* and *spindle-E* [23]. Thus, PIWI is not required for OSK and VASA localization during oogenesis but is required for maintaining their localization during embryogenesis. An alternative hypothesis is that PIWI, like Aubergine, also regulates patterning genes but that this function is redundant. This hypothesis, however, does not explain the fact that neither ectopic expression nor overexpression of PIWI causes a detectable defect in posterior patterning.

Given the association of PIWI with DCR-1 and dFMRP, the PIWI-mediated regulation is likely via the miRNA but not the siRNA mechanism, which is DCR-2-dependent, or the rasiRNA mechanism, which does not depend on either DCR-1 or DCR-2 [33, 41]. This hypothesis is further supported by the similar phenotypes observed in embryos depleted of PIWI, DCR-1, and dFMRP but not DCR-2. It is possible that PIWI might bind to novel small RNAs to achieve this function, given recent findings that mammalian PIWI subfamily proteins bind to PIWI-interacting RNAs (piRNAs) [66–70]. If so, these novel RNAs must function in a DCR-1-dependent pathway in the cytoplasm given PIWI's localization to the cytoplasm in early pole cells. The function of the PIWI/DCR-1-mediated miRNA or novel small-RNA pathway in germline specification is very similar to that of other germ-cell regulators, such as *gcl* and mtlrRNAs [60, 62], in that these genes are required for pole-cell formation but not for abdominal segmentation. However, unlike embryos from the *gcl-bcd* females [71], embryos from the *piwi-bcd* females exhibit no cell-cycle delays in the anterior nuclei and no significant changes in the morphology of anterior nuclei (data not shown). Furthermore, GCL mediates a transcriptional repression mechanism [72]. Thus, the effect of the PIWI-miRNA mechanism on pole-cell formation may be distinct from the *gcl*-mediated mechanism.

It is important to note that the PIWI-mediated miRNA pathway positively regulates the expression of OSK and VASA, in contrast to the known translational repression role of the miRNA pathway. In support of this observation, the PIWI ortholog in the mouse, MIWI, also appears to positively regulate gene expression, likely by enhancing mRNA stability and translation [73, 74]. Alternatively, it is possible that PIWI regulates an unidentified intermediate protein whose function is to repress the expression of OSK and VASA.

Conclusion

We previously discovered that *piwi* is essential for the self-renewal of adult germline stem cells in *Drosophila* [46]. Recent studies have demonstrated that the miRNA pathway is involved in division and self-renewal of adult germline stem cells in the *Drosophila* ovary [42, 75]. Our study reported here further connects PIWI and the miRNA pathway and reveals their crucial role in germline fate determination during embryogenesis. These observations suggest that the germline and stem cells may share a common miRNA-mediated mechanism in defining their fates. Given the high degree of conservation of the miRNA machinery during evolution, this pathway may function in diverse organisms in determining the germline and stem cell fates.

Experimental Procedures

Sucrose-Gradient Fractionation of PIWI Complexes and Western Blotting

Embryos (0–2 hr after egg laying) were collected from *TAP-piwi* and w^{1118} flies and lysed by being homogenized in polysome lysis buffer

(100 mM KCl, 5 mM $MgCl_2$, 10 mM HEPES, 0.5% NP-40, and 1 mM DTT), 1× complete mini-EDTA protease inhibitors (Roche, Indianapolis, IN), and either 80 U/ml of RNaseOUT or 10 μg/ml of RNase A. The lysate was spun for 15 min at 15, 000 rpm at 4°C in a microcentrifuge. Two mililiters of the clarified lysate (50% buffer and 50% embryos) was loaded onto continuous 15%–50% sucrose gradients supplemented with 2 μM EDTA and 15 mM Mg acetate, plus 40 U/mL of RNaseOUT. The gradients were centrifuged for 3 hr at 150,000 g (35,000 rpm) in the SW41 rotor (Beckman Coulter, Fullerton, CA). Gradient fractions were collected manually, and 25 μl of each fraction were assayed by western-blot analysis with the following antibodies: rabbit anti-VASA [76] (1:2000), rabbit anti-calmodulin-binding peptide (1:2000, Upstate Biotechnology Co., Lake Placid, NY), and rabbit anti-dFMRP, anti-DCR-1, and anti-DCR-2 (1:1000, gifts from G. Hannon). For the assay of *piwi's* dosage effect on OSKAR and VASA, 50 0–2 hr embryos of each fly genotype were lysed in 200 μl of 2× SDS sample buffer, and 20 μl of each fraction was assayed by western-blot analysis with the following antibodies: rabbit anti-OSKAR (1:2000, gift from Paul Lasko), rabbit anti-VASA [76] (1:2000), and rabbit anti-actin (1:2000, Santra Cruz Biotechnology, Santa Cruz, CA).

Immunoprecipitation

TAP-PIWI was immunoprecipitated directly from relevant sucrose fractions with rabbit calmodulin-binding peptide antibody and Protein G Sepharose beads (Amersham Biosciences, Piscataway, NJ). Immunoprecipitates were washed with NT_2 buffer (150 mM NaCl, 1 mM $MgCl_2$, 50 mM Tris [ph 7.4], 0.05% NP-40, and 1 mM DTT), eluted from the beads in SDS buffer, and assayed by western-blot analysis with the above antibodies.

Generation of Embryos Depleted of Maternal PIWI, DCR1, DCR-2, or dFMRP

Embryos depleted of maternal *piwi, dcr1,* and *dfmri* were generated as previously described [77]. For more detailed information on the genetic crosses, see the Supplemental Experimental Procedures.

Pole-Cell Analysis

Embryos depleted of maternal PIWI, DCR-1, DCR-2, or dFMRP were generated as described above. Embryos with a 2X (wild-type) dose of maternal PIWI, DCR-1, DCR-2, or dFMRP are from *OreR* or w^{1118} females. For the creation of embryos with 4X maternal PIWI, *a P[myc-piwi]* transgene on a wild-type second chromosome was homozygosed; two such lines were examined for their effects on embryonic development. For embryos with 6X of maternal PIWI, *P[myc-piwi]* transgenes on the X and second chromosome were homozygosed to generate four transgenic copies in addition to the two endogenous copies on the second chromosome. Embryos with 2X maternal OSK were from *OreR* females. For embryos with 4X maternal OSK, an *osk* transgene was homozygosed on the second chromosome in the wild-type background as described in Ephrussi and Lehmann [50]. For pole-cell counts, all embryos were stained with VASA antibody, and the pole cells of 50 embryos of each maternal genotype described above were counted in triplicate for each embryo. When necessary, embryos were examined under Normarski optics so that pole-cell number could be assessed. The pole-cell counts were conducted at cycle 14 (cellular blastoderm). Embryo staging was performed as described in Zalokar and Erk [78] according to the number of nuclei present.

Supplemental Data

Supplemental Results, Experimental Procedures, one table, nine figures, and two movies are available online at http://www.current-biology.com/cgi/content/full16/19/1884/DC1/.

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